advantages and importance of CE in conjunction with mass spectrometry have also become appreciated. Analytical chemists are faced with the challenge of increasing sample complexity and decreasing sample quantities. Because of the complexity observed with most biological mixtures, there continues to be a need for the development of a highly efficient separation technique in conjuction with a sensitive and specific detector. The low quantities of analytes often available require nanoseparation techniques. The mass spectrometer is a selective and broadly applicable detector for analytical separations. It can provide information regarding the structure of unknown components present in a sample mixture with high specificity and sensitivity. The coupling of CE with MS combines the extremely high-resolving power and structural information in one system. Like any other separation technique such as GC–MS and LC–MS, the principal advantage of CE–MS is that analytes are identified by both their differential separation and their molecular masses and/or fragmentation patterns. An analytical separation that precedes MS analysis is often necessary to assure correct interpretation of the mass spectral data.

Fast, high-efficiency separation techniques are becoming ever more important in the race to discover new drugs. The potential complexity of libraries produced by automated parallel synthesis, combinatorial and genetically manipulated natural product chemistries are driving many developments in separation sciences.

CE, CEC and nano-LC are all potential candidates for such analyses and each has a requirement for a fast, sensitive detection system.

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Capillary Electrophoresis–Nuclear Magnetic Resonance

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Miniaturization is an important current trend in separation science and the development of capillary electrophoresis (CE), capillary HPLC (cHPLC) and capillary electrochromatography (CEC) are milestones in this respect. The electrophoretic techniques especially can achieve rapid and efficient separations using only very small volumes and they have become research tools with widespread applications. The advantages of this miniaturization are obvious: less sample is

required, less solvent is consumed, and the separation times are shorter.

The second trend in separation science is toward information-rich detection modes. Although UV-VIS fluorescence, and electrochemical detectors provide sensitive and simple detection, the information is generally not sufficient for unequivocal characterization or structural elucidation of compounds. For this purpose the coupling of capillary separation methods with electrospray mass spectrometry (ESI–MS) has proven to be highly successful. ESI–MS is an extremely sensitive detector and in many cases information about mass and fragmentation gives detailed structural information.

The enormous sensitivity gain in nuclear magnetic resonance (NMR) spectroscopy in recent years also enables the wider application of directly-coupled HPLC–NMR. NMR spectroscopy is considered one of the most powerful methods for determining chemical, dynamic, and spatial structural properties of organic compounds. Its capability to distinguish between most structural, conformational, and, in special cases, optical isomers is highly complementary to the information gained from MS experiments. NMR spectroscopy is nondestructive and spectra are recorded in solution. A combination with additional detectors is possible, e.g. HPLC–UV–NMR, HPLC–MS–NMR.

The miniaturization of liquid chromatography (LC)–NMR has additional advantages. The small volumes of eluent consumed in capillary separation techniques make the use of fully deuterated solvents economically feasible. Therefore, problems associated with protonated solvents are prevented. NMR solvent suppression techniques which can lead to distortion of parts of the spectra are no longer necessary. Thus, the entire range of the ^{1}H-NMR scale can be used in one- and two-dimensional experiments. The supplementary benefit of conserving material is particularly interesting for valuable samples, such as natural products or labelled proteins.

However, the NMR spectrometer is one of the least sensitive of all possible LC detectors. The miniaturization of the LC–NMR coupling for the application of capillary separation techniques requires a reduction of the detection volume by a factor of approximately 1000 in comparison to conventional LC–NMR systems. On these conditions, the techniques seemed to be incompatible. Despite these problems, because of the great potential of the technique, efforts have been made in the past few years to enable coupling of capillary techniques to NMR spectroscopy. To date, two different experimental approaches have been developed and evaluated. They differ mainly in the type of radiofrequency (rf) coil that is used for the NMR detection. This variation leads to different strengths and weaknesses of the two systems, which allow or hinder their use for particular applications.

Set-up for CE–NMR Coupling

The design of an interface for the coupling of capillary separation techniques with NMR spectroscopy is simple in principle. Since phase transfer is not necessary, detection can be carried out in the fused-silica capillary, which is used for CE separation. The detection takes place on-column similar to a common UV detection. The capillary is built into an NMR probe head. Even the inlet and outlet vials of the CE can be incorporated inside this probe, but for practical reasons to allow easy sample loading, it is more useful to retain the inlet outside the NMR spectrometer. In any case both vials are maintained at the same height to avoid siphon flow. The separation equipment, e.g. power supply or HPLC pump, have to be placed outside the magnetic field. High voltage is applied to one end of the capillary and the other vial is grounded. With vials outside the magnet (inlet vial or both vials) a capillary length of approximately 150–200 cm is required. This means the capillary is three to four times longer than a common CE capillary.

The limiting factor of the whole system is the low sensitivity of the NMR spectroscopy. The parameters that affect the detection limit are the type and size of the radiofrequency coil, the detection volume, and the so-called filling factor, the ratio of coil and sample volume. A optimal detection cell fills the purpose-build microcoil completely. Furthermore, the inner diameter of the used fused-silica capillaries have to fulfill the chromatographic requirements. Especially for electrophoretic techniques, the inner diameter of the capillaries is therefore limited ($\leq$100 μm) and the size of the coil has to be adapted to these requirements.

Two different microcoil NMR configurations have been used for online coupling of capillary separation techniques with NMR spectroscopy. One approach is based on a solenoid rf coil wound directly on the CE capillary (**Figure 1**). The detection volume for the system is thereby determined by the inner diameter of the capillary and the length of the coil. With 75 μm capillaries and a typical coil length of approximately 1 mm, detection volumes of 5–8 nL are obtained. Solenoid coils are positioned in a horizontal direction in the NMR spectrometer, perpendicular to the main magnetic field. The other approach uses saddle-type rf coils (**Figure 2**). The detection unit consists of a coil fixed onto a glass tube, into which the capillary is inserted. Due to technical problems, the reduction of

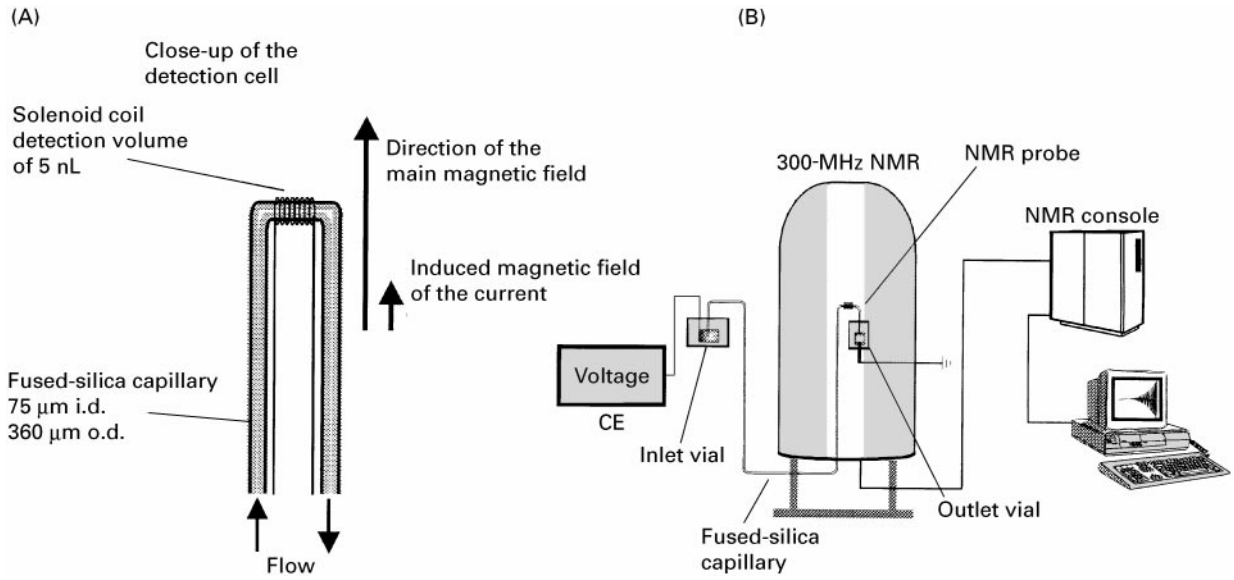

Figure 1 (A) Solenoid NMR interface. (B) Instrumentation of the solenoid NMR probe system. Adapted with permission from Gfrörer *et al.* (1999) *Analytical Chemistry News and Features* 71: 315A–321A, and Olsen *et al.* (1999) *Analytical Chemistry*, 71: 3070–3076.

the coil diameter is restricted. The smallest available coils have diameters of 1.5–2.0 mm and a length of 5–9 mm. To optimize the filling factor, special NMR capillary cells have been fabricated to a predetermined size by etching a standard fused-silica capillary with a HF solution only in the detection region. While the inner diameter in this part was widened, the rest of the capillary remained nearly the original diameter of 75 μm allowing the application of electrophoretic separation techniques. By varying the duration of etching, detection volumes between 180 and 440 nL were obtained. In contrast to the solenoid coil, the saddle coil is situated vertically in the NMR spectrometer, parallel to the main magnetic field.

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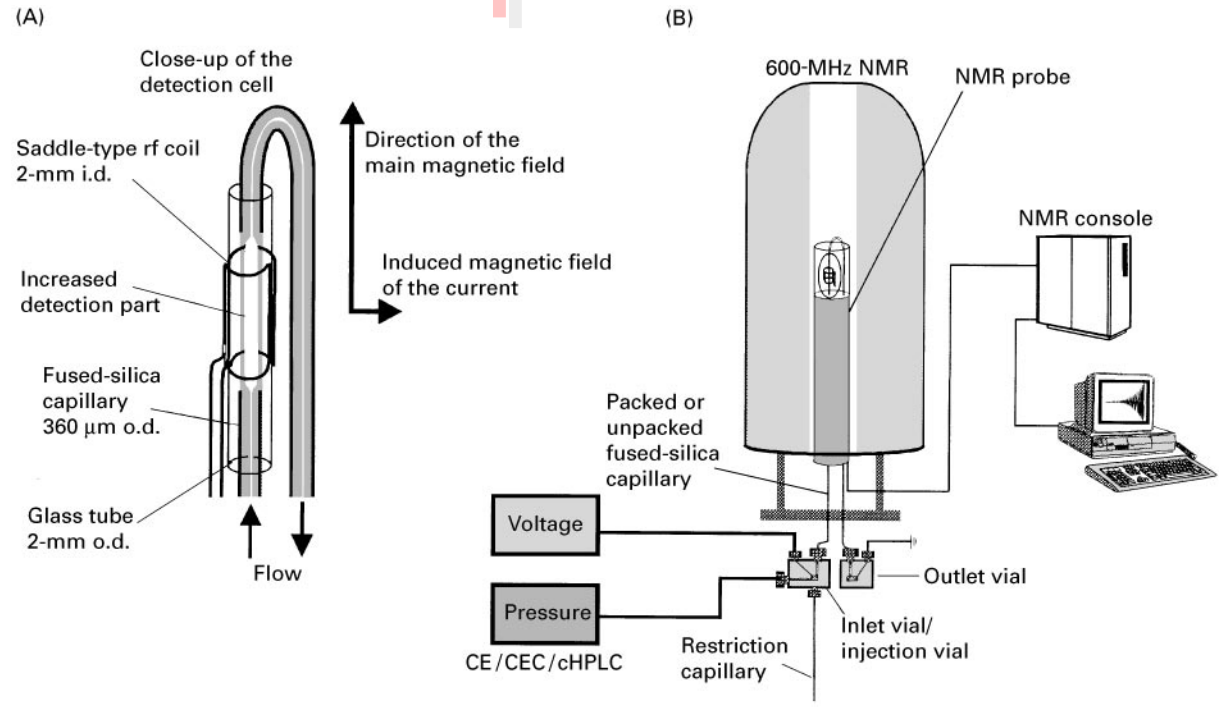

Figure 2 (A) Saddle-type NMR interface. (B) Instrumentation of the saddle-type NMR probe system. Adapted with permission from Gfrörer *et al.* (1999) *Analytical Chemistry News and Features* 71: 315A–321A.

Development of the Solenoid NMR Probe System

In 1994 Wu *et al.* described the first capillary electrophoresis NMR system with a solenoid rf microcoil. The coils were produced by winding copper wire around fused-silica capillaries with inner diameters of 75–530 μm. The resulting NMR detection cells had volumes of 5–200 nL. The possibility of an online NMR detection was shown for CE separation of amino acids. The set-up was not suitable for routine operation, e.g. each sample had to be loaded externally, after which the probe was inserted. The structural information of the NMR spectra was limited due to large line widths (7–200 Hz). The electrophoretic current had a clear effect to the NMR line width.

Further developments of this solenoid system focused on the NMR detection of mass limited samples. In 1995, Olson *et al.* presented a new microcoil design. Several modifications were devised to obtain higher resolution. The fabrication of the coil was slightly changed and the outer diameter of the capillary was increased (357 μm o.d., 75 μm i.d.). The main improvement was the reduction of the effect of magnetic susceptibility caused by the proximity of the rf coil to the sample. A perfluorinated organic liquid was used to match the susceptibility of the surrounding medium to that of the coil material. This lowered the static magnetic field inhomogeneities in the sample and thus improved resolution and line shape (line width < 1 Hz). As a consequence of the improved resolution, the sensitivity of the system increased. Limits of detection in the range of 100 pmol were reported for arginine, sucrose and a seven amino acid peptide using a 5-nL detection cell.

In 1998 Subramanian *et al.* presented solenoid microcoil probes for direct or inverse ^{13}C-NMR detection. Heteronuclear NMR techniques are an important component in determining full-structure information on unknown compounds. Due to the low relative sensitivity of ^{13}C-NMR, the detection volume was increased in comparison to the previous design. Capillaries with inner diameters of 700 μL were used for the fabrication of the probe. The resulting detection cells had volumes of 550–1200 μL. The natural-abundance ^{13}C-NMR limit of detection was below 100 nmoL. One-dimensional ^{13}C-NMR spectra and two-dimensional ^{1}H–^{13}C inverse-correlation NMR spectra were acquired using samples in the tens of micrograms range.

In 1999, Olson *et al.* applied the optimized NMR interface for CE–NMR coupling. Arginine, glycine and triethanolamine were used as model compounds to investigate in more detail the influence of the electrical current on the NMR signals. It was confirmed that for geometries, in which capillary and static field are not parallel, the electrophoretic current induces a magnetic field, which degrades the spectroscopic information obtainable from the CE–NMR spectra. To circumvent this effect, the electrophoretic voltage was periodically interrupted to obtain high resolution spectra. In addition, different sample-loading techniques including field-amplified stacking for sample preconcentration, were evaluated. Flow profiles were observed by the detection of the water signal of the loaded samples.

Development of the Saddle-type NMR Probe System

First results of this approach were reported by Behnke *et al.* in 1996. The LC capillary was mounted inside a modified NMR microprobe equipped with a 2.5-mm double-saddle Helmholtz coil. In static NMR experiments, a line width of 3 Hz could be achieved in 75-μm i.d. capillaries. However, this arrangement adversely affected the filling factor of the system and thus the sensitivity using this configuration was reduced significantly. First, coupling experiments with this system were performed with cHPLC. In comparison with CE, cHPLC provides a higher sample capacity and offers the possibility of peak preconcentration by the application of gradient elution. Online and stopped-flow NMR experiments of dansyl amino acids were carried out in 315-μm i.d. capillaries.

The sensitivity of the saddle-type system was improved by Schlotterbeck *et al.* in 1997. Decreasing the inner diameter of the rf coil from 2.5 to 2 mm improved the filling factor and thus the sensitivity and the line shape of the system. For online cHPLC-NMR experiments, the inner diameter of the capillary was increased to 180-μm i.d. in the detection region and this led to a limit of detection of 150 pmol. The feasibility of the interface was proved by its application to vitamin derivatives. The structure of a so-far unknown kitol, a retinyl acetate dimer, was determined from one- and two-dimensional ^{1}H-NMR spectra in both continuous and stopped-flow measurements.

In 1998 the configuration was modified for the application of electrophoretic techniques by Pusecker *et al.* The most important alteration was a purpose-built CE–NMR capillary. This capillary had a detection cell with an inner diameter of 190 μm corresponding to a detection volume of 240 nL, whilst the rest of the capillary still had the usual CE diameter of approximately 75 μm. CE–NMR measurements proved that for the saddle geometry, in which capillary and static magnetic field are parallel, the

spectroscopic information is not degraded by a magnetic field gradient induced by the electrophoretic current. The suitability of the configuration for electrophoretic methods was investigated by the application of CE and CEC–NMR spectroscopy to model systems. The favourable capabilities of the CEC–NMR coupling in view of the increased sample capacity and separation efficiency were demonstrated for the separation of five alkyl benzoates.

The optimization of the filling factor by a further increase of the detection volume to 440 nL improved sensitivity and line shape. A line width < 0.8 Hz was reported by Schewitz *et al*. The system was applied to the analysis of mixtures of pharmaceuticals and drug metabolites, nucleotides, peptides, natural products and ingredients of soft beverages. The possibility of CE stopped-flow NMR experiments was shown for an adenosine dinucleotide. The flow was halted by switching off the voltage at a retention time corresponding to the first NMR signals of the dinucleotide. By means of this stopped-flow mode, it is possible to accumulate spectra for a longer period of time. This enables the acquisition of two-dimensional NMR experiments. These techniques are substantial in determining full-structure information on unknown compounds. As an example, the two-dimensional TOCSY NMR spectrum of the adenosine dinucleotide is shown in **Figure 3**. In this spectrum cross-peaks indicate those protons which are coupled to each along an unbroken chain of couplings. Hence the coupling of each desoxyribose is clearly observable in two separate spin-coupling connectivities.

In 1999, a final improvement was made by Gfrörer *et al*. who coupled a gradient-elution CEC–NMR system to the interface. As an example, **Figure 4** shows the CEC–NMR separation of paracetamol metabolites extracted from human urine. The result is viewed as a contour plot with the CEC separation time on the vertical axis and the chemical shift on the horizontal axis. The peaks that spread throughout the figure arise from the residual water and acetonitrile in the deuterated solvents of the buffer. In addition, sets of peaks related to paracetamol and endogenous material are observed. Those marked (1) can be assigned to the paracetamol glucuronide. Clear identification is possible via the diagnostic shifts of the glucuronic acid at $\delta = 3.8$ and 4.2. The component marked (2) is the paracetamol sulfate. The NMR spectrum of the third compound is consistent with the endogenous material hippurate (3). The appropriate individual rows taken from the contour plot are shown on the right-hand side of **Figure 4**.

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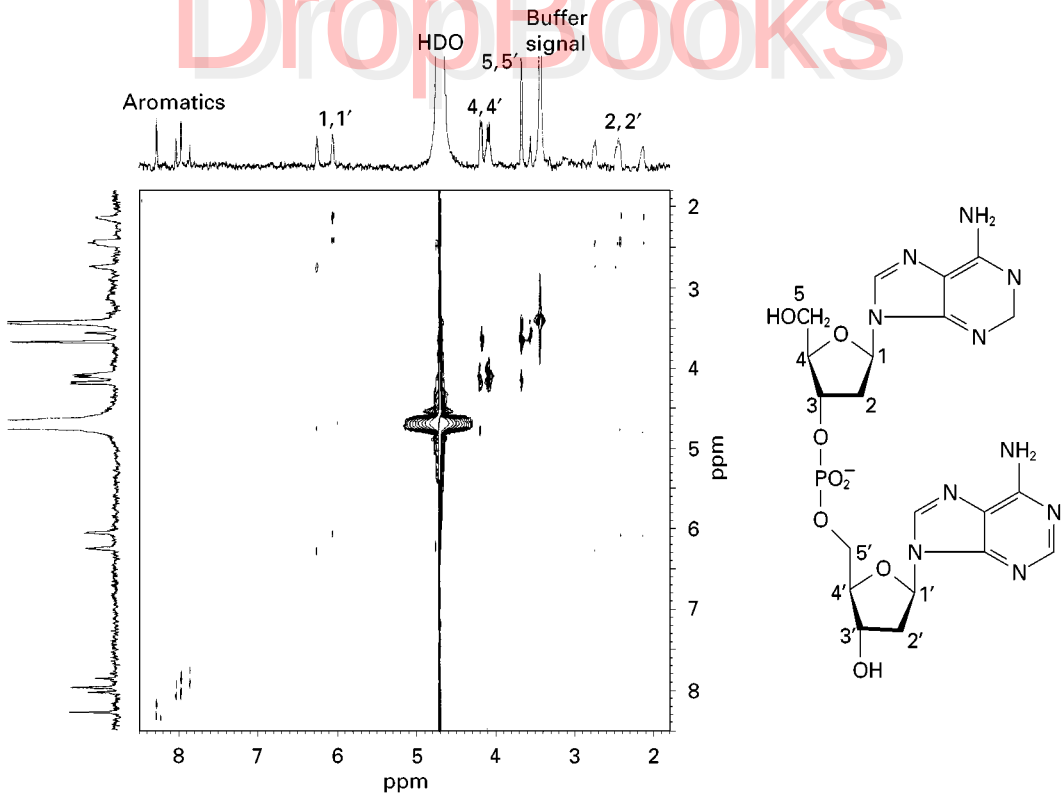

Figure 3 Two-dimensional CE ^{1}H–^{1}H-TOCSY NMR spectrum of adenosine dinucleotide. Adapted with permission of Schewitz *et al.* (1999) *Chromatographia* 50: 333–337.

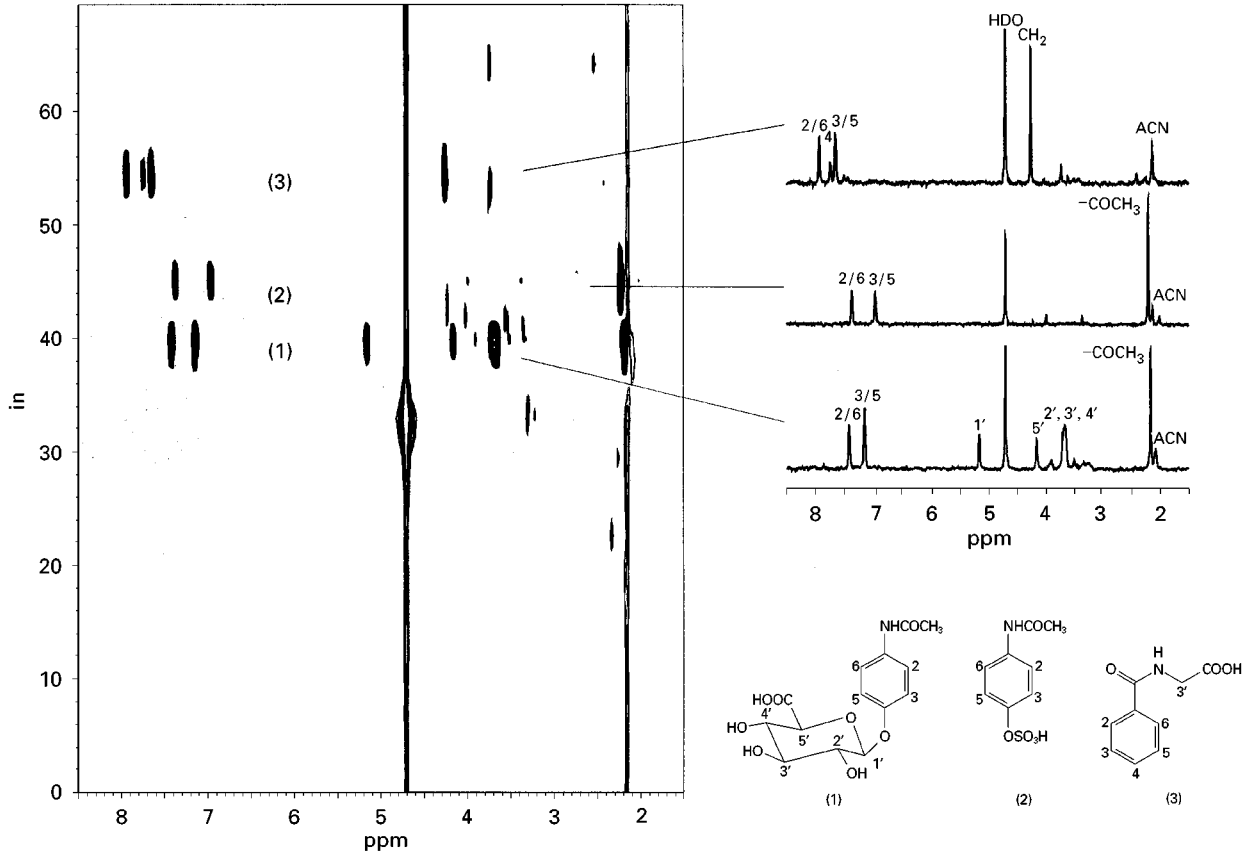

Figure 4 On-flow contour plot of the ^{1}H-NMR detected CEC separation of human urine. ^{1}H-NMR spectra on the right side are extracted from the contour plot. (1) Paracetamol glucuronide; (2) paracetamol sulfate; (3) hippurate. Adapted with permission from Pusecker *et al.* (1998) *Analytical Communications* 35: 213–215.

Comparison of the Systems

Both types of NMR detection system have strengths and weaknesses. The substantial advantage of the solenoid system is its better sensitivity. The low detection limit resulting from the perfect filling factor of the system allows extremely small detection volumes. This is important for electrophoretic separations, where the peaks often have volumes in the order of a few nanolitres and larger detection cells thus limit separation efficiency. It is obvious that the arrangement is well suited for mass limited samples, but unfortunately the small detection volume is offset by the need for high sample concentrations. Typically, these have been >30 mM but in some examples concentrations of >50 mM were necessary, and this then leads to a limitation in the number of possible applications. The approach may need special sample injection techniques like the field-amplified stacking.

Another problem of the solenoid CE–NMR set-up is the complex interdependence on flow rate, electric field and current. The current, which passes through the capillary, produces a magnetic field gradient that perturbs the uniformity of the main magnetic field of the NMR spectrometer. Large NMR line widths and reduced structural information are the consequences. High-resolution NMR spectra are only obtainable by periodic stopped flow capillary electrophoresis when the interruption of the current enables the acquisition of NMR spectra. However, this technique makes the already long separation times even longer.

The most important advantage of the saddle system is its easier handling capability. Already there is a large number and a wide range of reported applications to support this assertion. There are several reasons. Firstly, as described above, the capillary is arranged vertically in the saddle-type probe. Because the shim systems used for the field homogeneity adjustment in cryomagnets are optimized in the vertical z-direction parallel to the magnetic field, the shimming procedure is facilitated in comparison to the solenoid system. Furthermore, the coil is not permanently attached to the separation capillary, thus allowing the capillary to be easily exchanged for different applications without the risk of damaging

the rf coil. Finally the distance between coil and sample is relatively large and so problems with susceptibility do not occur.

On the other hand, the size of the saddle coil is problematic. Due to technical problems, it is not possible to reduce the coil diameter beyond a certain limit. To obtain a suitable filling factor, the detection cell has to be enlarged and the resulting detection volumes of 180–440 nL are much larger than in conventional CE and might be thought to lead to lowered separation efficiency of electrophoretic techniques. However, the fabrication method of these NMR cells produces cell profiles that avoid mixing and turbulent flows and thus the separation efficiency is not seriously compromised. Assuming a sufficient separation of the components large detection cells can even have advantages. Longer residence times of the components allow an improvement in the signal-to-noise ratio of the NMR spectra through the accumulation of an increased number of scans and the higher level of solvent reduces the necessary sample concentration to < 10 mM.

The two systems are complementary in approach and both have made contributions to a better understanding of the challenges involved in coupling CE and NMR. A decision on which type will find more widespread application will be determined by future improvements of the systems and possibly by the type of applications to which this technology will be applied.

Future Developments

Numerous obstacles to coupling capillary separation techniques with NMR spectroscopy have already been overcome. In the short term, the development is likely to be focused on the improvement of the NMR interface and a goal must be implementation of the advantages of solenoid and saddle systems. An interface that combines sensitivity and small detection volumes of the solenoid arrangement with the easy handling of the saddle-type configuration and thus allow routine and automated operations will find extensive use in the future.

One way to reach this goal might be the application of the new cryo-probe technology to capillary NMR spectroscopy. Such probes already exist for conventional NMR spectroscopy and provide, in favourable circumstances, an improvement in signal-to-noise ratio of approximately 400%. This is achieved by cooling the rf coil to reduce the level of thermal noise. This might increase the sensitivity of a saddle coil by a substantial factor without the need of a size reduction.

Another improvement would be integration of UV detection directly into the NMR probe. This would allow the performance of real stopped-flow measurements and this would, for example, circumvent the problem of NMR line broadening by the electrical current for the solenoid interface.

Usually, capillary separation science techniques are optimized to handle minimal amounts of sample. However, because of the desirability of direct combination with NMR spectroscopy, new technologies have to be developed that allow an effective separation of relatively large sample amounts in small volumes without a reduction of the efficiency. There are many possible improvements, which are necessary in order to increase the capabilities of this new hyphenated technique. With forthcoming advances in the sensitivity of NMR spectroscopy, CE–NMR and CEC–NMR will become practical and useful methods in situations which require separation and structural determination of components of mixtures in severely mass-limited situations.

See also: **II/Chromatography: Liquid:** Electrochromatography; Nuclear Magnetic Resonance Detectors. **Electrophoresis:** Capillary Electrophoresis; Capillary Electrophoresis-Mass Spectrometry. **III/Clinical Applications:** Capillary Electrophoresis.

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